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Viewpoint

Why only time will tell

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Abstract

The nematode *Caenorhabditis elegans* has become a model system for the study of the genetic basis of aging. In particular, many mutations that extend life span have been identified in this organism. When loss-of-function mutations in a gene lead to life span extension, it is a necessary conclusion that the gene normally limits life span in the wild type. The effect of a given mutation depends on a number of environmental and genetic conditions. For example, the combination of two mutations can result in additive, synergistic, subtractive, or epistatic effects on life span. Valuable insight into the processes that determine life span can be obtained from such genetic analyses, especially when interpreted with caution, and when molecular information about the interacting genes is available. Thus, genetic and molecular analyses have implicated several genes classes (*daf*, *clk* and *eat*) in life span determination and have indicated that aging is affected by alteration of several biological processes, namely dormancy, physiological rates, food intake, and reproduction.

1. Introduction

Aging research is a vast field of scientific endeavour: a PubMed search with the keyword 'aging' yields 125 000 entries. Recently, a small number of these entries

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(218) can also be found with the keyword 'elegans' because the nematode *Caenorhabditis elegans*, a classical model organism, is now being used more frequently to investigate the biology of aging. This is part of a general impetus to obtain insight into the molecular mechanisms of aging by harnessing the power of genetic analysis in invertebrate and unicellular model systems, including the budding yeast *Saccharomyces cerevisiae* (148 entries) and the fruitfly *Drosophila melanogaster* (818 entries).

Here, in an attempt to give an overview of what is known about genes and processes that affect life span in worms, a series of independently standing appendices are presented. We have tried to be concise rather than exhaustive and we apologize to the people whose work was not included because of space limitations. In contrast to the appendices, which summarize the current state of knowledge, the main text is concerned with trying to discern the strengths and the limits of the genetic approach when applied to the study of aging. We have drawn all our examples from *C. elegans*, in keeping with the present authors biases, and because the genetics of aging is well developed in this organism.

2. *C. elegans* as a model organism for aging research

A number of characteristics make the worm particularly suitable for the study of aging. It is small ($\sim$1 mm as adult) and thus large numbers of animals can be grown and manipulated. As it is short-lived ($\sim$15 days for the wild type), aging experiments can be completed in a relatively short time. Also, worms can be frozen indefinitely in liquid nitrogen such that a large number of genetically distinct strains can be created and stored for future use at almost no cost. This also means that unwanted genetic changes in a strain can be kept to a minimum as it is possible to return to the original frozen strain if changes occur. *C. elegans* is an internally self-fertilizing hermaphrodite. This is a particularly valuable characteristic as it means that *C. elegans* tolerates almost total homozygosity. Thus, isogenic strains can be easily obtained and the effect of mutations of interest can be studied with a minimum of interference from other loci. *C. elegans* is also capable of cross-fertilization by males, which makes it possible to perform genetic crosses. Finally, the worm is extremely hardy and resistant to genetic sickness. In fact, under the conditions in which it is grown in the laboratory, the worm does not have to move to feed or to reproduce. These characteristics are important because all mutations known to date to affect aging, including those that prolong life span, have other phenotypic effects, which can sometimes be severe.

3. Life span as a phenotype

It is difficult to define aging except by saying that it inevitably results in death. Thus, the best measure of aging is the increase in the probability of death of the organism with time. It is legitimate, therefore, to score life span as the main characteristic when attempting to study genes that affect aging.

As a phenotype, life span is fickle because it is cumulative, and is therefore very sensitive. This sensitivity makes it powerful as it allows one to score even very small physiological differences between two strains as a difference in life span. But this sensitivity is also a problem as worms are mostly cultured under relatively ill-controlled conditions. They are grown on agar plates on a lawn of *E. coli*. The agar mix contains various nutrients for the bacteria, such as yeast extract, tryptone or peptone, whose composition is not precisely defined and is likely to vary from batch to batch. In addition to the agar, anything that affects the bacteria will indirectly affect the worms, such as any genetic change in the standard bacterial strain, or the age of the bacterial lawn at the time at which it is used as food for worms. Moreover, although worm cultures are kept in temperature-controlled incubators, one cannot ensure absolute temperature uniformity, especially over a lifetime of weeks where the accumulated effect of even small differences in temperature are likely to result in measurable differences of life span. These problems are partially overcome by the inclusion of numerous control strains in every experiment and the practice of carrying out repeat experiments. (Our experience is that at least three independent experiments with a sample size of 50 for each are necessary to obtain a reliable measure for life span differences of less than 50%.) The finding of a relative difference in life span between strains scored at the same time is therefore a much more meaningful measure than absolute differences in life span observed among separate experiments, particularly when the experiments are conducted in different laboratories.

3.1. Life span and genetic background

Another consequence of the special nature of life span as a phenotype is its extreme sensitivity to genetic background. There are many reasons to suspect that life span is a highly polygenic trait, and indeed most theories of aging support this view. Although one of the strengths of *C. elegans* as a model system is the possibility to obtain highly isogenic strains, potential differences in genetic background should still be considered and precautions should be taken to minimize them. Indeed, in a recent study we carried out to investigate aging in mutants that affect nervous system and muscle function (Lakowski and Hekimi, 1998), we have found that a large proportion of strains harboured background mutations that were not responsible for the main phenotype of the strain but that nonetheless affected life span. Repeated backcrossing of the mutant strain to the canonical wild type strain (N2) removed additional mutations in all cases but one.

3.2. Long lives

The great power of *C. elegans* for aging research is that it is a genetic system in which mutations can readily be identified. In fact, a number of mutations that increase life span are being studied. It is an inescapable conclusion that the normal activity of a gene must limit life span in the wild type when a loss-of-function mutation of the gene results in an increased life span of the mutant.

3.3. Short lives

It is much more difficult to interpret mutations that shorten life span because one cannot a priori distinguish between mutations that increase the rate of a life span-limiting process and mutations that produce a pathology whose incidence does not normally increase with age. We believe that this is not a semantic distinction. One can easily imagine structures or processes that never fail within the life span of wild-type animals, or do not fail more often with chronological age. In fact, the existence of mutant strains whose average and maximum life span is several times longer than that of the wild type implies the existence of highly resistant structures which can support a life span longer than that of the wild type (Kenyon et al., 1993; Larsen et al., 1995; Lakowski and Hekimi, 1996, 1998). Indeed, such long-lived mutants are often anatomically very similar to the wild type. This means, for example, that the worm's collagenous cuticle, which serves as both a mechanical support and a protection from the environment, is capable of resisting environmental assaults for much longer than ever needed in the wild type. A mutation that would make the cuticle less resistant to the point of shortening the mutants' life span is unlikely to identify a function that identifies one of the causes of normal aging.

The considerations above, however, do not mean that all genes whose loss-of-function phenotype results in a shorter life are irrelevant to aging. From examples in *C. elegans*, we will see that with careful genetic analysis and prudent interpretation of epistatic relationships, some short-lived mutants can help to unravel the molecular mechanisms of aging.

3.4. Big and small effects

The range of life-span increases brought about by single mutations is quite wide. For example, *daf-2* mutants can live more than two times longer than the wild type (Kenyon et al., 1993), while *clk-1*, *-2* and *-3* mutants live only approximately 30% longer than the wild type (Wong, et al., 1995; Lakowski and Hekimi, 1996). Mutants that live very long are understandably easier to study. They can allow for the unambiguous identification of epistatic or suppressor mutations (Kenyon et al., 1993; Larsen et al., 1995; Taub et al., 1999). For example, mutations in either *daf-16* or *ctl-1* dramatically shorten the life span of *daf-2* mutants. In fact, the life span of these double mutants is as short or even shorter than that of the wild type, while mutations in *daf-16* and *ctl-1* shorten the life span of the wild type only slightly.

On the other hand, that mutations in single genes lengthen life span only modestly is not a clear indication of the importance of these genes for life span determination. For example, mutations in *clk-1*, which increase life span relatively little, can double the life span of *daf-2* mutants under some environmental circumstances (Lakowski and Hekimi, 1996). Similarly, double mutants of *clk-1* with *clk-2* or *clk-3*, live from two to three times longer than the wild type (Lakowski and Hekimi, 1996). Another way to say this is that genes function only

in the context of other gene activities: a *daf-2* mutant lives shorter than the wild type in a *daf-16* mutant background and a *clk-1* mutant lives two times longer than the wild type in a *clk-2* mutant background. Thus, it appears that, although finding mutants that increase life span is a prerequisite to identify processes that are important for normal aging, the magnitude of the effect of a given gene mutation cannot be directly interpreted.

3.5. Environmental effects

In the laboratory, *C. elegans* can be grown under a variety of conditions at temperatures ranging from 15 to 25°C. Most commonly, for aging experiments, worms are cultured on a dense lawn of *E. coli* on a solid substrate (agar). On dense bacterial lawns, the pumping action by which the worm ingests bacteria is efficient. Worms can also be grown under liquid conditions with either living bacteria or bacteria killed by heat or irradiation, or in defined axenic medium, that is, entirely without bacteria. Liquid culture is stressful for the worms as it makes it more difficult for them to ingest food. This probably leads to caloric restriction (Appendix C), which would explain why most genotypes live longer when grown in liquid culture (Braeckman et al., 1999). For some genotypes these conditions are too stressful as many mutants cannot be grown at all in liquid culture or in axenic medium. For example, *clk* mutants cannot grow in axenic medium and are infertile in medium with killed bacteria (Braeckman et al., 1999).

The different conditions under which worms can be grown raises the question of how to interpret differences of life span of a given genotype under different environmental conditions. It is well known that temperature affects aging, and this is true for wild-type as well as for mutant worms. Some genotypes live long only at some temperatures. For example, *clk-2*(*qm37*) is a temperature-sensitive embryonic lethal mutation at 25°C (Hekimi et al., 1995), and *daf-2* mutants arrest permanently at a specific developmental stage at 25°C (Kenyon et al., 1993). On the other hand, *daf-2* mutants live a very long time when first grown at a permissive temperature that allows them to complete development, and then transferred to 25°C for their adult life. Clearly, *daf-2* affects a process that limits life span, yet given that *daf-2* is also required for this process to proceed normally, conditions (25°C) can be found where the absence of *daf-2* is clearly deleterious and leads to permanent developmental arrest.

Similarly, when grown on bacterial plates, the life span of *clk-1* is only marginally affected by *daf-16* (Lakowski and Hekimi, 1996, 1998). In fact, it appears to be shortened exactly by the amount by which *daf-16* also shortens life span in a wild-type background. However, in liquid culture *daf-16* mutations limit much more severely the life span of *daf-16*; *clk-1* double mutants. We believe, however, that this cannot be interpreted to mean that *daf-16* is required for the long life of *clk-1*. Indeed, we have argued above that it is very difficult to interpret the meaning of short life spans because they can always be the result of a life-shortening pathology that does not limit life span in the wild type. Therefore, when a particular double mutant combination is short-lived only under particular environ-

mental conditions, this cannot be used to interpret the genetic meaning of the interaction. The fact that an environmental condition can be found in which it is deleterious to be simultaneously a *clk-1* mutant and a *daf-16* mutant, so that the life span of the double mutant is shorter than that of *clk-1*, does not tell one much about either gene, given that, under different conditions (bacterial plates), *daf-16*; *clk-1* double mutants live long. The only way in which the shorter life span of the double mutant under the harsher conditions could be meaningful is if *clk-1* increased life span in liquid culture by a different mechanism than by which it increases life span on bacterial plates, only the former requiring *daf-16*.

4. Genetic interactions

As illustrated in the previous section, one way to gain insight into the function of genes that affect life span is to study their interactions with other genes and with each other by constructing and characterizing double mutants. This has been done in a number of studies, some of which are described in detail in the appendices. Here we examine the classes of possible outcomes when double mutants are constructed (Fig. 1), and discuss how much one can or cannot conclude from such studies.

4.1. Additivity and synergism

Some double mutants live longer than any of the two component single mutants. The extension of life span in double mutants may arise from two types of genetic interactions: additivity or synergism. When the life span of the double mutant is approximated by adding the percent life span increases over wild type of the single mutants, then the effect of the single mutations is additive. Any increase much larger than an additive increase can be defined as synergistic. It is unclear at the present time whether additive and synergistic interactions have different meanings.

An example of synergism is provided by the interactions of the three *clk* genes: double mutants of any pair of *clk* genes live much longer than the single genes (Lakowski and Hekimi, 1996). Such an outcome suggests that the two mutations increase life span by mechanisms that are at least partially different from each other. In other words, the gene products do not function in a linear cascade of biochemical interactions. They might impinge on the same downstream process but in manners that are sufficiently distinct that the process is altered more severely when both mutations are present (see also Appendix A).

Fig. 1. The study of double mutants reveals different types of interactions between genes that affect life span in the nematode *C. elegans*. In each of the five illustrated cases, the life span of a given strain is indicated by the position of a schematic worm along the horizontal axis. The genotype of the strain is given just below the worm. In every case, a black worm represents the wild type, a large red worm represents the double mutant, and the small green or blue worms represent the constituent single mutants. The relationships between life spans are schematized and not scaled. The functional significance of these interactions is discussed in detail in the text (Section 4).

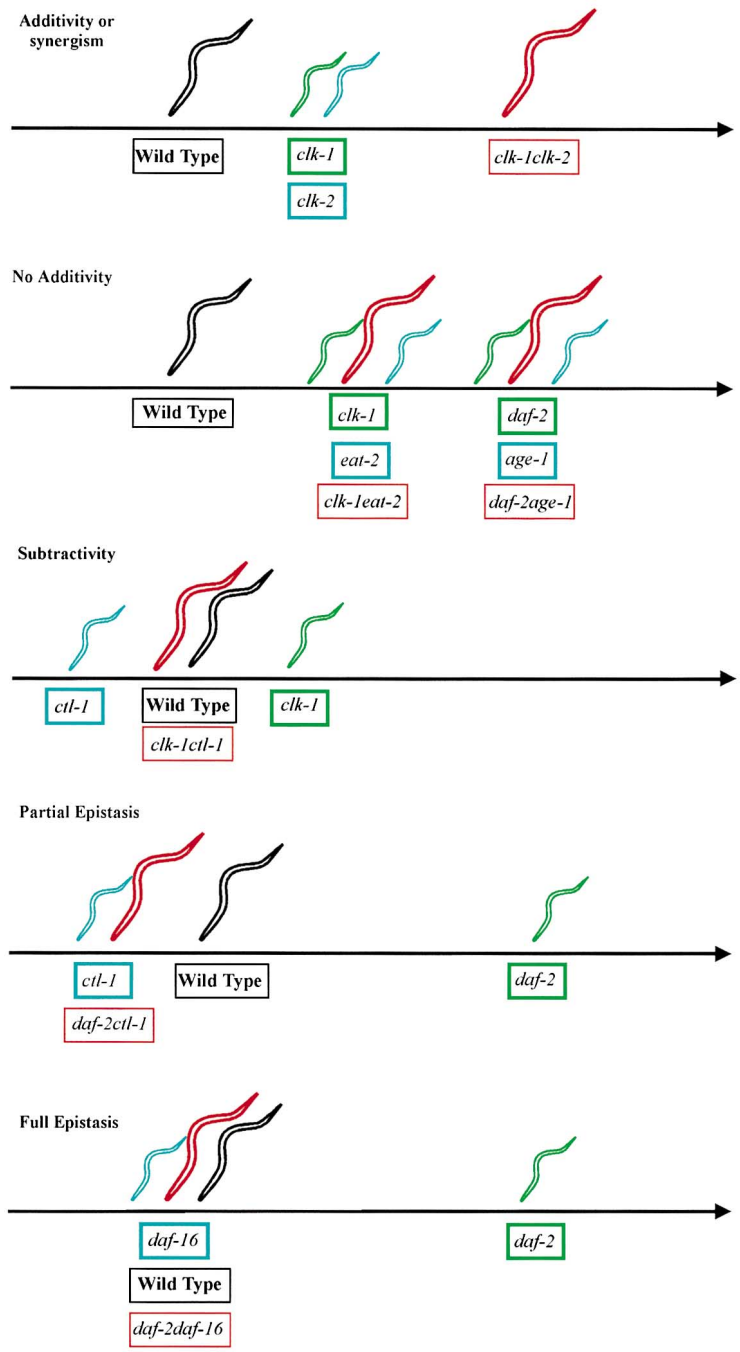
Additivity or synergism
Wild Type
clk-1
clk-2
clk-1clk-2
No Additivity
Wild Type
clk-1
eat-2
clk-1eat-2
daf-2
age-1
daf-2age-1
Subtractivity
ctl-1
Wild Type
clk-1ctl-1
clk-1
Partial Epistasis
ctl-1
daf-2ctl-1
Wild Type
daf-2
Full Epistasis
daf-16
Wild Type
daf-2daf-16
daf-2

Fig. 1.

4.2. Absence of additivity

Another possible outcome is that the double mutant lives no longer than any of the component single mutants. For example, *eat-2*(*ad465*); *clk-1*(*e2519*) double mutants live no longer than either *eat-2*(*ad465*) or *clk-1*(*e2519*) which produce a similar increase in life span (Lakowski and Hekimi, 1998). Except for life span, the phenotypes of these two mutants are very different from each other. *clk-1* mutants are slow developing and slow behaving, but display a fully wild-type appearance and morphology (Wong et al., 1995). However, *eat-2* mutants, which have reduced food intake due to slow ingestion of bacteria, develop and behave at essentially wild-type rates (except for food intake) but appear transparent and morphologically stunted (Avery, 1993). The two mutations are fully additive except for life span, so that the double mutants are both slow growing and slow behaving, and appear transparent and stunted. These observations have been interpreted as meaning that *clk-1* and caloric restriction impinge on the same downstream process that affects life span (Lakowski and Hekimi, 1998) (Appendix C). Another interpretation could be that the double mutant might not succeed in living longer than it does because of novel deleterious effects produced by the combined effects of the two underlying mutations. This is an example of how the absence of positive effects on life span is generally difficult to interpret.

The interpretation of an absence of additivity can be more straightforward in some cases. For example, double mutants of *daf-2* and *age-1* do not live longer than either of the single mutants (Dorman et al., 1995). However, the interpretation that they impinge on the same process is much more clear than in the case of *clk-1* and *eat-2*. Indeed, *daf-2* and *age-1* mutants have the same dauer constitutive phenotype and are placed at the same level of the genetic pathway of dauer formation (Appendix B). Furthermore, both genes have been cloned and shown to encode gene products (an insulin-growth-factor like tyrosine kinase transmembrane receptor and a PI3-kinase, respectively) that are known to interact in several systems. Here, the absence of additivity, together with other data, indicates that the life span increase in both mutants is due to altered regulation of the dauer formation pathway, and not to some unknown consequences of mutations, unrelated to the normal functions of the gene products in the wild type.

4.3. Subtractivity

The occurrence of subtractivity is best illustrated by the interaction of *clk-1* with *ctl-1* (Taub et al., 1999). *ctl-1* mutants display decreased levels of catalase activity and have a shortened life span compared to the wild type. However, the degree of shortening of the life span of *clk-1* by *ctl-1* (the life span of the double is 73% of the life span of *clk-1*) is very similar to the degree to which *ctl-1* shortens life span in a wild-type background (76%). Here it is the shortening of life span in one case, and the lengthening in the other, that are additive, which suggests that the mechanisms by which *clk-1* and *ctl-1* impinge on life span are substantially different. However, because a shorter life span is observed in the double mutant, it

is not possible to conclude very firmly. Indeed, one cannot exclude the possibility that *ctl-1* is one of the genes that are necessary for the life span extending activity of *clk-1*. Even so, it must not be the only target as the disruption of *ctl-1* only partially abrogates the life span extension conferred by *clk-1* mutations. Furthermore, the *clk-1*(*e2519*) mutation extends significantly the life span of *ctl-1*; *daf-2*, which in a *clk-1* (+) background lives no longer than *ctl-1* mutants (Taub et al., 1999) (Section 4.4, Appendix C).

4.4. Epistasis for life span

A mutation is epistatic to a second mutation when the phenotype of the double mutant is indistinguishable from that conferred by the first mutation. One can look at the epistatic relations between mutations by considering only one of the phenotypes of the mutants. For example, when only life span is considered, then *ctl-1* appears to be fully epistatic to *daf-2*. Indeed, *ctl-1* fully suppresses the dramatically increased life span of *daf-2*, so that the double mutant lives as short as *ctl-1*. However, *ctl-1* suppresses none of the other phenotypes of *daf-2* mutants, in particular, their dauer constitutive phenotype. These results strongly suggest that *ctl-1* is required for the long life of *daf-2* but do not indicate that the long life of *daf-2* is actually caused by an action of *daf-2* on *ctl-1*(+). In other words, because a short life span is observed, it is possible that the conjunction of the mutations in both genes is deleterious enough to produce a shortened life span without having impinged on the process that *daf-2* affects to prolong life span. It should be noted, however, that in this particular case, a closer link between the actions of *daf-2* and *ctl-1* is likely as various dauer constitutive mutants have been found to be stress resistant and have elevated levels of enzymes that detoxify oxygen radicals (Larsen, 1993; Honda and Honda, 1999; Taub et al., 1999) (Appendix D). Furthermore, *ctl-1* mutants have lower catalase levels (Taub et al., 1999).

4.5. Full epistasis

The interaction between *daf-16* and *daf-2* is one of the cleanest cases of epistasis (Kenyon et al., 1993). The phenotype of the double mutant is indistinguishable from that of *daf-16* mutants, for life span as well as for all other phenotypes (Gottlieb and Ruvkun, 1994). For example, the double mutants are incapable of forming dauer larvae as is *daf-16*. This suggests very strongly that the effect of *daf-2* on life span is mediated by *daf-16*. Consideration of the molecular identities of the genes clarifies the situation even further: DAF-2 is a transmembrane receptor in a classical signal transduction pathway and DAF-16 is a transcription factor (see Appendix B).

As we have seen above, most interactions are not as clear cut as those that can be observed between *daf* genes, whether *daf-2* and *age-1*, or *daf-2* and *daf-16*. One possibility why this might be so is that the *daf* genes function in a signal transduction pathway. Their biochemical activities might not directly impinge on aging, as might repair or detoxifying enzymes, for example. Rather, their action

results in a downstream signal that likely has an effect on a large number of targets. One might venture to predict that interactions between the targets of *daf-16* will not show such beautifully clear genetic interactions. In addition, the picture becomes more complicated when other *daf* genes are taken into consideration. For example, *daf-12*, which encodes a nuclear hormone receptor and acts in parallel to the insulin-like signaling pathway, shows allele-specific interactions with *daf-2* with respect to both dauer formation and life span (Larsen et al., 1995; Gems et al., 1998)

4.6. Allele-specific interactions

The study of allele-specific interactions can reveal different types of interactions between genes. For example, two sets of *clk-1 clk-2* and *clk-3 clk-1* double mutants were constructed: one set with the strong allele *clk-1*(*qm30*), and another set with the much weaker allele *clk-1*(*e2519*). As described above, interactions between the *clk* genes are all additive or synergistic. However, allele-specific interactions are very different for *clk-2* compared to *clk-3*. *clk-3*(*qm38*); *clk-1*(*qm30*) mutants are much more severe than *clk-3*(*qm38*); *clk-1*(*e2519*) mutants, including for development, life span and general health (Lakowski and Hekimi, 1996). This is the expected result if *clk-1* and *clk-3* have partially overlapping functions: in the absence of *clk-3*(+) the phenotype becomes more severe as the activity of *clk-1* is more reduced in the more severe allele *qm30*. In contrast, the double mutants with *clk-2*(*qm37*) containing either *e2519* or *qm30* are identical for development as well as for life span. This suggests that *clk-2* functions either downstream or upstream of *clk-1* and that the residual activity of *e2519*, which makes it a weak allele of *clk-1*, depends entirely on the presence of a wild-type copy of *clk-2*.

5. Conclusions

The viewpoint we have tried to defend here is that the aging process can be studied genetically in a model organism such as *C. elegans*, which allows one to identify mutations that prolong life span and to quickly carry out genetic experiments. We believe that the identification and study of long-lived mutants will be crucial to understanding aging as such mutations necessarily identify processes that normally limit life span.

Beyond this, more elaborate questions can be asked and answered by performing classical genetic analyses with these mutants. Yet, it is clear that life span is an unusual phenotype, and that some of the interactions we can observe have to be interpreted with great caution. This is because all genes serve functions that contribute to the viability of the organism, and because life span studies measure the occurrence of death. Altering the function of a gene may lengthen life span under particular conditions, but will invariably contribute to make the organism more susceptible to death under some other genetic or environmental conditions.

It is also clear from the examples we have given that knowledge of the molecular identity of aging genes can greatly enhance our ability to interpret the outcome of genetic interactions. One might think that when molecular functions are known genetics becomes less important. To the contrary, we believe that molecular identities are particularly precious when they contribute to a coherent picture of the meaning of genetic interactions. Only the links between phenotype and molecular function provided by genetics can identify the processes that are indeed causal to aging.

Acknowledgements

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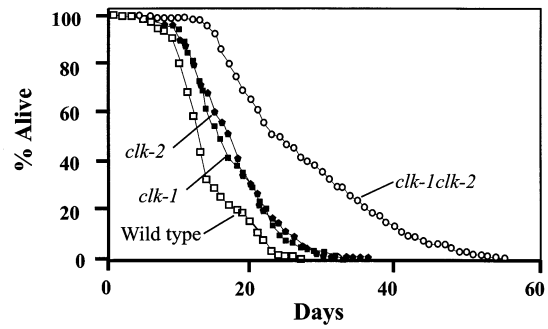
Appendix A. The *clk* genes

Several biological processes have been found to influence the life span of the nematode *C. elegans*, including dormancy, food intake, and physiological rates[1]. A group of at least four *clk* genes, namely *clk-1*, *clk-2*, *clk-3*, and *gro-1* has been proposed to affect life span by altering the latter[2]. Mutations in any one of the *clk* genes produce a complex phenotype that includes the average slowing down of development and rhythmic behaviors, as well as the lengthening of life span[2,3] (Fig. 2A, B). For example, at 18°C, all Clk mutants have a mean life span that is at least 3 days longer than that of the wild type[2]. While the increase of the duration of post-embryonic development of *clk* mutants contributes to their increased mean and maximum life span, it is not sufficient to account for the increase in life span[2].

clk double mutants exhibit a very pronounced increase in the duration of development and life span (Fig. 2A, B). For instance, the *clk-1 clk-2* double mutant takes approximately 4 days longer than the wild type to develop and lives 14 days longer at 18°C. The synergism of the effects of individual *clk* mutations on life span indicates that they affect different molecular processes. However, they all appear to extend life span by altering the same biological process, i.e. physiological rates. Indeed, there is a strong positive correlation between the duration of development and adult life span for both the wild type and strains carrying mutations in one or two *clk* genes (Fig. 2C). It is reasonable to speculate that life span is determined, at least in part, by the interplay between damage accumulation and repair. Thus, slower physiological rates in *clk* mutants (illustrated by slower developmental rates) should produce less damage, and the slow life of the *clk* mutants is probably causal to their long life. Consistent with this idea is the observation that increasing *clk-1* activity by overexpression of the wild-type gene accelerates physiological rates and shortens life span[4].

A



B

Genotype	Duration of post-embryonic development in days	Life span in days (mean ± S.E.)
Wild type (N2)	2.5	14.9 ± 0.3
clk-1(e2519)	3.0	18.4 ± 0.4
clk-2(qm37)	3.0	18.7 ± 0.5
clk-1(e2519) clk-2(qm37)	6.0	28.2 ± 0.9

C

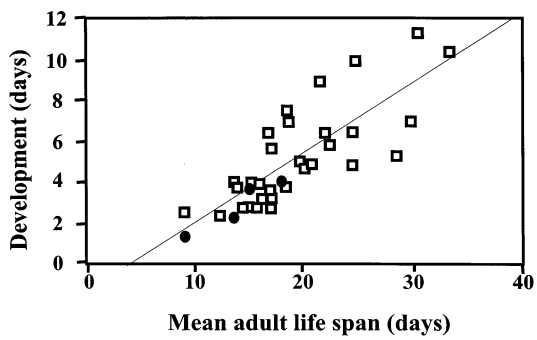


Fig. 2. *clk* mutations interact to determine life span in *C. elegans*: (A) Survival curves of *clk-1*(*e2519*) and *clk-2*(*qm37*) single and double mutants at 18°C. The graph shows the percentage of worms alive on a given day after hatching. *clk-1 clk-2* double mutants live longer than *clk-1* and *clk-2* single mutants; (B) The length of development (from hatching to adult molt) and the mean life span at 18°C are given for the genotypes presented in A; (C) The relationship between the length of post-embryonic development and mean adult life span at different temperatures. Circles represent the wild type and squares represent different mutant strains containing *clk-1*, *-2*, *-3* mutations, either singly or in double mutant combinations. The positive correlation between post-embryonic development and adult life span indicates that the effect of *clk* genes on development and life span is similar to the effect of lowering temperature. This suggests that *clk* mutants live long because they live slowly. (After data in Lakowski and Hekimi[2]).

Mutations in another group of genes, the dauer formation genes *daf-2* and *age-1*, also extend life span (Appendix B). The *daf-2* and *age-1* mutations alter life span in a manner that is different from that of the *clk* mutations by at least two criteria. First, while the life span extension of both *daf-2* and *age-1* depends on the activity of another gene, *daf-16*, loss-of-function mutations in *daf-16* cannot suppress the long life of *clk mutants*. At most, a slight reduction of life span is observed in *daf-16*; *clk-1*/*-3* double mutants, that corresponds to the life-shortening effect of *daf-16* on wild-type background[2,5]. Second, *daf-2* and *clk-1* mutations display a synergistic effect on life span: *daf-2 clk-1* double mutants live much longer than either of the single mutants and can live over five times longer than the wild type.

The *clk-1* gene has been cloned and encodes a small protein of unknown biochemical function that is well conserved, both phylogenetically from proteobacteria to eukaryotes[1], and functionally[6,7]. Indeed, both worm and rat sequences can rescue the phenotype due to mutations in *coq*7, the yeast homologue of *clk-1*. *coq*7 mutants are defective in the synthesis of ubiquinone, an electron carrier that is required for respiration, and therefore cannot grow on non-fermentable carbon sources[8]. CLK-1 protein localizes to the mitochondria in all the cells of the worm[4] and in yeast, where it has been shown to be part of the inner mitochondrial membrane[9]. However, the level of respiration is only very mildly affected in *clk-1* mutants compared to the wild type. It has been hypothesized that *clk-1* could be implicated in the cross-talk between the mitochondria and the nucleus to regulate gene expression as a function of the level of energy metabolism in the cell[4,10].

A.1. References (Appendix A)

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Appendix B. The insulin-like signaling pathway and aging

In *C. elegans*, a neurosecretory-signaling pathway regulates the decision to continue growth or to enter an alternative, developmentally arrested larval stage termed the dauer stage. Animals enter the dauer stage when environmental conditions do not favor growth and reproduction, such as when food resources are low and population density and temperature are high. Dauer animals exhibit numerous unique traits including increased resistance to environmental stress and a life span that well exceeds that of adult animals[1]. Components of this neurosecretory pathway include sensory neurons, hormone-like signaling molecules as well as signaling cascades to transduce signals in the cells of downstream target tissues[2]. Numerous mutations that enhance or suppress dauer formation have been isolated and these have been ordered into a complex, branched genetic pathway based on genetic epistasis. There appears to be at least three partially independent signaling pathways that regulate dauer formation including a TGF-β like pathway, a cyclic nucleotide-signaling pathway and an insulin-like pathway. Interestingly, the insulin-like signaling pathway appears to regulate longevity as well as dauer formation.

Mutations in the gene *daf-2*[3], result in constitutive larval arrest at the dauer stage (at restrictive temperatures) and a 2-fold increase in life span (under more permissive conditions)[4]. *daf-2*, which encodes an insulin receptor-like tyrosine kinase, transduces signals through *age-1*[5] a phosphatidylinositol-3-OH kinase family member, which generates the 3-phosphoinositide second messengers PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 (Fig. 3). These in turn activate *akt-1* and *akt-2*[6], which are believed to directly antagonize the forkhead transcription factor *daf-16*[7,8]. Mutations that decrease signaling in this pathway, such as mutations in *daf-2* and *age-1*, result in upregulation of *daf-16*, leading to an increase in dauer arrest and to a dramatic extension of life span. Mutations in *daf-16* suppress these dauer formation and long life span phenotypes.

There are at least twelve putative insulin-like signaling ligands in the *C. elegans* genome[9]. It is not currently known which particular insulin genes are involved and what processes regulate activation of the worm insulin-like pathway. Recent evidence suggests that mutations that affect ciliated sensory neurons, which have been previously shown to have a role in dauer formation, can regulate aging through the insulin-like signaling pathway[10]. In particular, mutants with defects in cilia structure, as well as animals in which sensory structures have been laser ablated, exhibit increased life span. These mutations could not enhance the long life span of *daf-2* mutants, indicating that they function in the same pathway. In support of this view, the long life span of the sensory neuron mutants is almost fully suppressed by mutations in *daf-16*. This suggests that ciliated sensory neurons

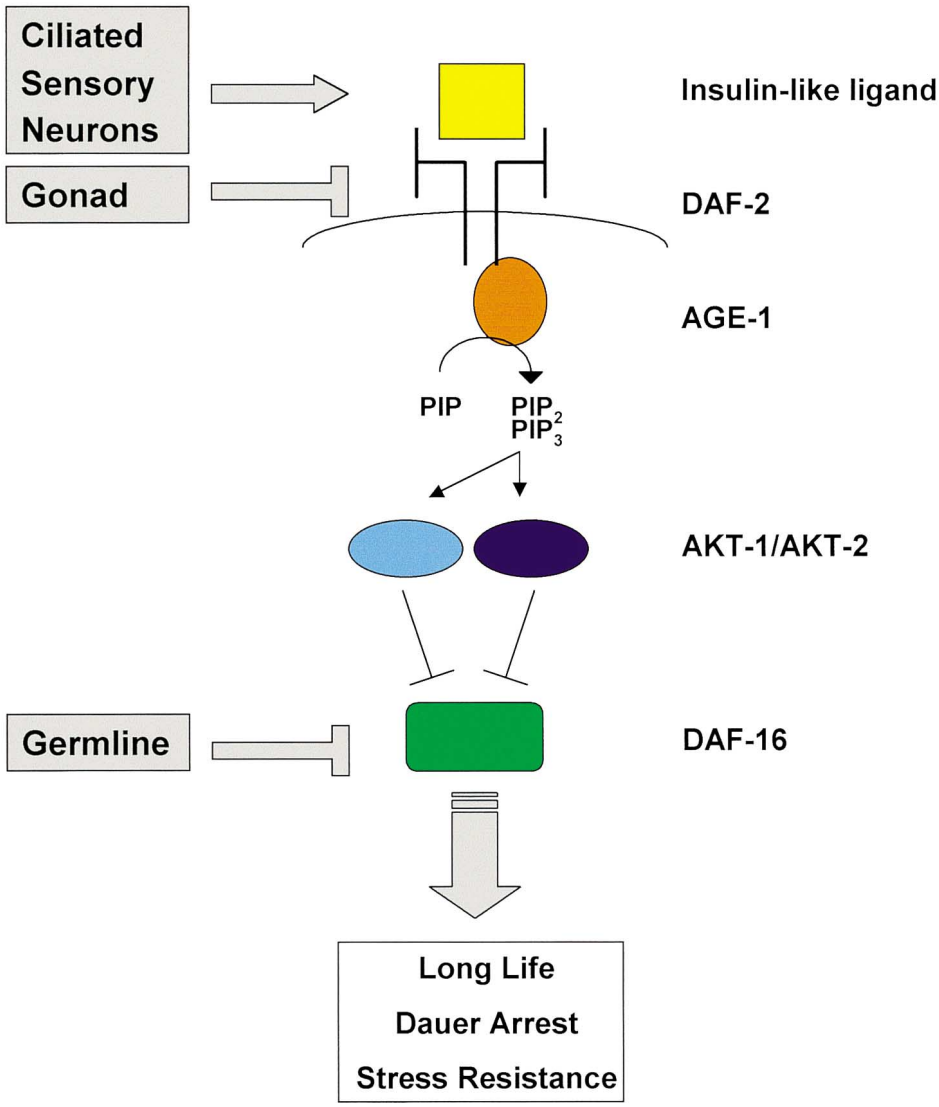

Fig. 3. A model of the *C. elegans* insulin-like signaling pathway and the various processes that modulate it. Under growth promoting conditions, an insulin-like signaling ligand activates DAF-2, which recruits AGE-1. This results in the production of the second messengers PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3, which activate AKT-1 and AKT-2, which in turn antagonize the forkhead transcription factor DAF-16. Absence of signaling through this pathway results in upregulation of DAF-16, increased dauer formation, and long life. The ciliated sensory neurons control an insulin-like signal that stimulates DAF-2 activity. The reproductory system produces mutually antagonistic signals. The gonad appears to inhibit DAF-2 signaling while the germ line suppresses DAF-16 activity.

may control the release of an insulin-like signaling molecule that regulates aging. In the absence of appropriate environmental cues or in the case of non-functional sensory neurons, the level of this ligand falls, resulting in decreased activation of the insulin-like pathway, and consequently in the upregulation of *daf-16* and increased life span (Fig. 3). In addition, two genes involved in Ca^{2+} -regulated secretion in neurons, *unc-64* and *unc-31*, have been demonstrated to enhance lifespan through the insulin-like signaling pathway[11]. These two genes may be directly involved in the release of an insulin-like signal. Together, these results suggest that neurons modulate the release of an insulin-like signal that regulates life span through the insulin-like signaling pathway.

A second process that appears to be involved in regulating the insulin-signaling pathway is the reproductive system. Hsin and Kenyon[1] propose a model in which the germline produces a signal that suppresses long life by antagonizing *daf-16* function (Fig. 3). Laser ablation of the germ line precursor cells (Z2 and Z3) releases this suppression and results in increased lifespan. Conversely, the gonad appears to produce a life span promoting signal that antagonizes *daf-2* insulin-like receptor function. In support of this view, whole gonad ablation in a *daf-16* mutant background, in which the germ line signal is blocked, results in a decrease in life span. It appears that these two opposing signals are balanced as removal of the whole gonad in wild-type animals has no effect on life span[4].

How upregulation of *daf-16*, the terminal output of the insulin-like pathway, increases life span is not known. However, it appears that upregulation of *daf-16* might activate some aspects of a dauer specific program, including genes that regulate metabolism as well as stress resistance (Appendices C and D).

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Appendix C. Caloric restriction

Over 60 years ago, it was discovered that reducing the caloric intake of rats could extend their mean and maximal lifespan. Caloric restriction (CR, also called dietary restriction) has since been found to extend the life span of a wide range of animals (reviewed in Weindruch and Walford[1]), and remains the only well-documented experimental means of extending vertebrate life span. The effects of CR have been best studied in rodents, where it has been shown that CR results in many physiological changes, including reductions in body weight and body temperature, blood glucose and insulin levels, and oxidative damage, and has also been found to boost DNA repair capabilities (reviewed in Sohal and Weindruch[2]). The mechanism by which CR increases life span is unknown, as it is unclear which of the many changes brought about by CR is/are responsible for the life-span extension. However, recent studies have shown that CR induces the production of growth factors and stress proteins, and that production of these proteins increases the resistance of the cells to stress and might help them cope with the adverse effects of aging and age-related disease[9,10].

Lowering food intake, in this case bacteria, has also been shown to increase the life span of *C. elegans*. Klass[3] showed that growing worms in liquid culture with reduced levels of bacteria increases their life span. Lakowski and Hekimi[4] showed that mutations in *eat* genes, which affect the function of the pharynx, the organ that pumps bacteria into worms, also increase worm life span. A number of observations suggest that these mutations do indeed prolong life span by restricting the food intake of worms. In particular, *eat* mutants have a 'starved' appearance, that is, they are more transparent and often smaller than the wild type[5]; mutations in some *eat* genes, like *eat-2*, actually affect the rate at which the animal pumps in food[5,6], and for some mutations (*eat-2* and *eat-6*) the life-span extension correlates with the severity of the feeding defect[4]. Moreover, *unc* mutations, which affect muscle or nervous system function, but not the pharynx, do not increase life span[4] (Fig. 4).

In addition to caloric restriction, there are at least two other distinct genetic mechanisms for extending life span in *C. elegans*. One mechanism involves genes

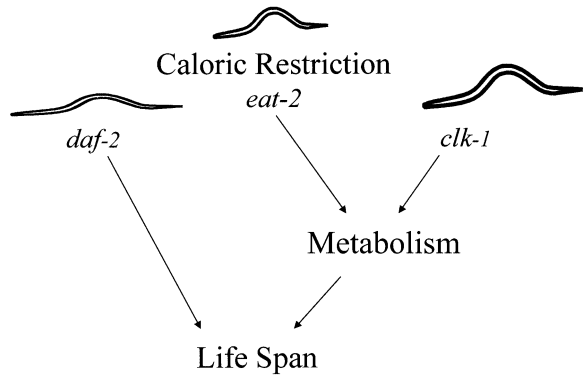

Fig. 4. Interactions between the different genetic mechanisms that extend life span in *C. elegans*. *daf* genes, such as *daf*-2, and *clk* genes, such as *clk*-1, extend life span by distinct mechanisms. Caloric restriction extends life span in a manner that is distinct from that of *daf*-2, but similar to that of *clk*-1. CR and mutations in *clk*-1 might both extend life span by affecting the metabolic state of the animal.

that regulate dauer formation, such as *daf-2* and *daf-16* (Appendix B); the other mechanism involves genes that affect physiological rates, the Clock genes, such as *clk-1* (Appendix A). Genetic interactions between *eat-2* and *daf-2*/*daf-16* suggest that dauer genes extend lifespan by a mechanism that is distinct from that of CR: *eat-2*; *daf-2* double mutants live substantially longer than either *eat-2* or *daf-2* mutants, and mutations in *daf-16* do not suppress the long life of *eat-2* mutants[4]. In contrast, genetic interactions between *eat-2* and *clk-1* suggest that *clk-1* mutations may extend lifespan by a mechanism similar to that underlying CR: *eat-2*; *clk-1* double mutants do not live significantly longer than animals that have only *eat-2* or *clk-1* mutations[4]. However, given the very different phenotypes of *eat* and *clk* mutants, it is unclear what process they both affect. One possibility is that CR and mutations in *clk-1* both affect the metabolic state of the animal. Although it is not yet clear, in worms or in other organisms, whether CR results in decreased metabolic rates, reduced caloric intake could result in a metabolic shift to lessen energy consumption, a possibility that has also been raised to explain the phenotype of *clk-1*[7,8].

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Appendix D. Stress resistance and ROS

The life span of an organism likely depends on a balance between internal and external damaging conditions and the capacity of the organism for resistance and repair. This hypothesis is being pursued with the nematode *C. elegans*, whose resistance to a number of external and intracellular stresses such as excessive temperature and reactive oxygen species (ROS) can be tested (for review see Lithgow[1] and Ishii and Hartman[2]).

Mutants with an extended life span such as *daf-2* and *age-1* are resistant to thermal stress[3]. *daf-2* and *age-1* function in the same signal transduction pathway that regulates dauer larva formation (Appendix B). In particular, *age-1* encodes a homologue of phosphatidylinositol-3-OH kinase (PI-3K). Interestingly, a synthetic inhibitor of PI-3K activity can increase thermotolerance in wild-type animals, as well as their mean life span by 10%[4]. Also, induction of thermotolerance by a transient temperature shift at the adult stage (3 h at 35°C) extends life span in wild-type worms by 14%, but by 30% in *age-1* mutants[3].

More generally, if a common battery of genes is implicated in the response to multiple stresses, one would expect that sensitization by a particular stress could render the animal more resistant to another type of insult. Indeed, wild-type worms that have experienced an oxidative stress display increased X-ray resistance[5]. Molecular chaperones of the *hsp* family and superoxide dismutase (*sod*) genes could be responsible for stress resistance, but the exact molecular mechanisms of this adaptive response remain elusive.

The activites of two enzymes, superoxide dismutase (SOD) and catalase, protect the cell from oxidative damage. SOD converts superoxide into hydrogen peroxide

and water, and catalase in turn produces oxygen and water from hydrogen peroxide. The activity of SOD certainly contributes to the dauer stress resistance traits[6], where it is increased 5-fold compared to 2 day old adults[7]. A positive role of SOD in lengthening life span has been proposed in *age-1*[7], and *daf-2* worms, where the expression of *sod-3* is upregulated and correlated with the DAF-2 phenotype[8]. It is interesting to note that overexpression of SOD can be sufficient to increase life span. For example, the overexpression of *sod-1* in motorneurons in *Drosophila* prolongs life span by 40%[9]. The role of catalase has also been explored. A mutant locus that reduces catalase activity (*ctl-1*) suppresses the long life of both *daf-2* and *age-1*[10] (Appendix B). An increase in catalase activity and expression of a cytosolic catalase with age has been reported for the *age-1* mutant[7,10] but is has not been described for *daf-2* mutants. However, contradictory evidence exists for a role of catalase in dauer larvae resistance traits. Indeed, as compared to young wild-type adults, *daf-2* dauer larvae exhibit an increase in catalase activity[10], while wild-type and *age-1* mutant dauer larvae do not[7].

Small molecules (salen–manganese complexes) acting as catalytic scavengers of ROS, have also been used to try to detoxify ROS[11]. Remarkably, the life span of wild-type worms is extended upon treatment with such molecules[12]. However, due to the high concentrations used in these experiments and the absence of dose-dependency, it is difficult to conclude that they act only by detoxifying ROS. Also, the relationship between life span and the products of oxygen-dependent metabolism has been further studied by the analysis of *mev-1* mutants[13]. Mutations in the gene *mev-1*, which encodes a mitochondrial enzyme (succinate dehydrogenase cytochrome b_{540}), affect electron transport from succinate to ubiquinone. The mean life span of *mev-1*(*kn1*) mutants is shortened to 8.5 days at 25°C in the presence of increased oxygen levels (60%), as compared to 13.0 days for the wild-type. Consistent with this observation, *mev-1* mutants are also hypersensitive to paraquat, a compound that generates oxygen radicals[13]. These phenotypes are probably caused by an oxygen concentration-dependent intracellular increase in ROS due to the mutation.

Finally, signal transduction proteins have also been implicated in stress response, as illustrated by the discovery of methuselah in *Drosophila*, a gene encoding a G-protein-coupled receptor[14], and by studies on *tkr-1* in worms[15]. In Drosophila, a partial loss-of-function mutation in *methuselah* causes a 35% increase in life span as well as resistance to starvation, high temperature and ROS. In worms, the putative receptor tyrosine kinase gene *tkr-1* lengthens life span by 65% when overexpressed, and induces resistance to heat and UV treatment[15]. The cascades of events triggered by these receptors remain to be determined.

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Appendix E. Aging and metabolism

The dauer larval stage of *C. elegans* is a developmentally arrested, alternative third larval stage that is entered under unfavorable environmental conditions. Dauer larvae live much longer than adults, and mutations in the genetic pathway that regulates dauer larva formation affect the aging process (Appendix B). These observations have prompted investigations into possible physiological and biochemical particularities of dauer larvae and dauer formation mutants.

The transition from egg to adult is normally accompanied by a switch in metabolism from the glyoxylate cycle to the tricarboxylic acid (TCA) cycle[1]. This

occurs relatively rapidly during the L2 larval stage and the major fuel source consequently changes from fat reserves to ingested matter. In dauer larvae there is a marked (10-fold) reduction in flux through the TCA cycle relative to young adults[2]. A concomitant reduction in oxygen consumption[3] (per dry weight), ATP levels[1], and mitochondrial oxidative capacity (3-fold) relative to young adults[2] has also been observed. The contribution of the glyoxylate cycle to dauer metabolism remains debated. Two reports have suggested that the key glycolytic enzymes malate synthase and isocitrate lyase are decreased[1,4], while another has observed a 3-fold increase of both components[5]. Intuitively one imagines that the dauer must subsist off its stored energy reserves, but whether catabolic routes other than the glyoxylate cycle are utilized to do this remains unclear.

Many genes that are normally ubiquitously expressed during other larval stages (such as actin, histones and ubiquitin), are heavily or completely shut down in the dauer larvae[6]. Conversely, key survival genes such as RNA polymerase (Rpo) I and III, which transcribe rRNAs and tRNAs, as well as genes that encode stress-resistance proteins such as *hsp-90*, *sod-3* and *ctl-1*, are maintained at high levels[6]. An intriguing finding has been the observation that in dauers, Rpo II activity, which normally transcribes mRNA, is depressed despite the presence of normal Rpo II protein levels. Heat shock rapidly overrides this block to induce a dramatic increase in stress-response mRNAs for genes such as *hsp-16* and *hsp-70*[6].

daf-2 and *age-1* mutants are defective in their ability to properly regulate dauer development and live a long time (Appendix B). A logical prediction might be that the increased life span observed in mutant adults may result from the expression of dauer-like metabolic properties. At least one study has observed elevated levels of malate synthase and isocitrate lyase in *age-1* and *daf-2* animals[5]. Conversely, levels of the TCA enzyme isocitrate dehydrogenase are not decreased in these animals and the levels of this enzyme did not decrease with age as it does in a control population[5]. Other studies have also found no significant decreases in the O_2 consumption levels of *age-1*, or indeed for any of the *clk* mutants[7,8] (Appendix A). Additional studies employing CO_2 production as a measure of metabolism corroborate the finding for *age-1*[9]. In the latter study, however, CO_2 production rates were found to be significantly reduced (4-fold) in both a *daf-2*(*e1370*) mutant and a *daf-2*(*e1370*) *clk-1*(*e2519*) double mutant. This is surprising because studies have placed *age-1* immediately downstream of *daf-2* (Appendix B), and the effect of these genes on life span or dauer formation are not additive (see main text). Thus, if the rate of oxidative metabolism of *daf-2* mutants is decreased, this is not causal to their long life.

An intriguing observation has suggested that both a *clk-1*(*e2519*) and, even more so, a *clk-1*(*e2519*) *daf-2*(*e1370*) double mutant have markedly elevated levels of total ATP relative to age-matched controls[7]. Given the hypometabolic phenotype of *clk* mutants (slow growth and slow reproductive rates), one possibility is that ATP production is intact in these animals but that ATP is used at a lower rate.

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